

Production-optimization of Biosurfactant from Mangrove Sediment Bacteria using Media Salinity, Differences in Carbon Source Concentration and pH Levels

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The research's goal is to find the best conditions for making a biosurfactant from bacteria by looking at the molecular structure of the biosurfactant made, the pH and salinity of the medium, and the amount of olive oil used as a carbon source. This research is descriptive/experimental research for screening and characterization of biosurfactants, while experimental research is for optimizing biosurfactant production. Optimization in this research used a completely randomized design (CRD). The parameter used in biosurfactant optimization is IE24. According to research on the bacterium *P. aestusnigri* NNA 0-9 from mangroves, the best conditions for biosurfactant production were using a 5% concentration of olive oil as a carbon source at pH 7 without the addition of NaCl and time incubation for 6 days. Based on the FTIR results, the biosurfactant molecular structure analysis shows that the compound that was made is rhamnolipid. The conclusions of this study provide a detailed description of the optimal parameters required for biosurfactant production while also providing identification of the molecular structure produced by the bacterium *P. aestusnigri* NNA 0-9 from mangrove. These findings provide a strong foundation for further development regarding the use of biosurfactants in industrial and environmental applications. In the optimization stage, statistical approach methods such as Response Surface Methodology (RSM) need to be used to obtain an optimization model.

Keywords: Biosurfactant, mangrove, optimization, *pseudomonas aestusnigri*, rhamnolipid.

INTRODUCTION

Amphiphilic compounds known as biosurfactants have the ability to lower the surface tension of liquids with varying polarity, including oil and water. Biosurfactants can be produced by microorganisms either extracellularly or as secondary metabolites from their cell walls. the bioavailability of hydrocarbons can be increased through the application of biosurfactants in bioremediation, which will quicken the pace of breakdown. Biosurfactants have several advantages over synthetic surfactants, including being biodegradable, biocompatible, low toxicity, having a variety of structures, and being stable under changing environmental circumstances. Biosurfactant-producing bacteria are a promising option for effective hydrocarbon remediation because of these benefits (Andry *et al.*, 2023).

Ambon is the capital of Maluku Province, which is a development center, so it has high shipping activity. Shipping activities in Ambon Bay, such as loading and unloading ships, docking ships, and cutting ship hulls, can cause oil spills at

sea (Buonocore *et al.*, 2020). An oil spill from the explosion of the MV Fu Yuan Fu F66 occurred in Ambon Bay. In 2018, there was an oil leak from the Pertamina Wayame Oil Fuel Terminal (TBBM), Teluk Ambon. High tides can eventually carry oil spills into mangrove wetlands (Figures 1 and 2).

Oil-contaminated areas may be home to bacteria that break down hydrocarbons and produce biosurfactants (Heryadi *et al.*, 2023).

Even though biosurfactants have been widely used, there are challenges in increasing production yields, namely adjusting production capabilities. How high the emulsification index (IE24) is can indicate the activity and quality of biosurfactants. Factors that influence biosurfactant productivity include carbon source concentration, pH, and salinity. For example, *Serratia* sp. ZS6 had the highest biosurfactant production when cultured with 2% olive oil as the carbon source. The use of olive oil as a carbon source has been widely reported to produce good biosurfactant activity on various bacterial species, so it was used in this research (Chebbi *et al.*, 2017). The pH factor also influences

Hamzah, A.H.P., D.Y. Heryadi, L. Judijanto, S.A. Pramono and N.C. Lestari. 2024. Production-optimization of biosurfactant from mangrove sediment bacteria using media salinity, differences in carbon source concentration, and pH levels. Journal of Global Innovations in Agricultural Sciences 12:391-398.

[Received 18 Jan 2024; Accepted 20 May 2024; Published 30 May 2024]



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biosurfactant production. For example, the emulsification ability of biosurfactants from *Bacillus subtilis* decreases at an acidic pH while increasing at a neutral pH. Another factor that influences biosurfactant production, especially in isolates from marine environments, is salinity. For example, biosurfactant from *Planococcus halotolerans* IITR55 isolated from the Yellow Sea had the highest production at 1% NaCl but was stable up to a concentration of 9.2% (Humaida *et al.*, 2018).

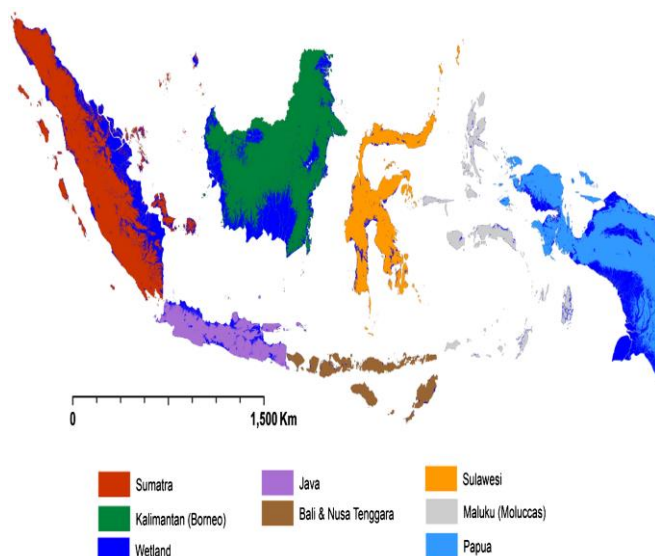


Figure 1. National wetlands in Indonesia, adapted from MARGONO *et al.* (2014)



Figure 2. Sample of mangrove forest in indonesia, adapted from Kusmana (2014).

Biosurfactants are made up of different molecules and parts, such as fatty acids, protein polysaccharides, glycolipids, flavolipids, phospholipids, lipopolypeptides, and flavolipids.

The diversity of biosurfactant structures depends on the strain and cultivation conditions. The structural composition of the molecules that make up a biosurfactant will determine its suitability for a particular application (Hamzah and Harijati, 2023). The isolates used in this research were petroleum-degrading bacteria from mangrove sediments. These isolates were screened, and the best isolates were used for optimization. Optimization and characterization data are expected to provide information for developing mangrove ecosystem management and bioremediation to deal with possible impacts from pollutants (Chen *et al.*, 2017).

Amphiphilic surface-active chemicals known as biosurfactants lower the surface tension between two immiscible phases. Much of the biosurfactant industry is produced by microorganisms. Microorganisms produce biosurfactants in an effort to modify the surface properties of cells, resulting in increased adhesion to pollutants or increased partitioning and bioavailability for microorganisms (Andry *et al.*, 2023). The structure of a biosurfactant is called amphiphilic because the two ends have different properties, namely that the tail group is hydrophobic (fatty acids and saturated or unsaturated hydrocarbon chains) and the head group is hydrophilic (mono-, in-, or polysaccharides, acids, peptides, anions, and cations). Biosurfactant in the water phase will form micelles if the concentration of biosurfactant in the water phase exceeds a threshold, also known as critical micelle concentration (Hamzah *et al.*, 2021).

Based on their structure, biosurfactants are grouped into several classes. The first class is the biosurfactant most commonly used in the industrial world, namely glycolipids. The most widely known example is rhamnolipid, generally produced by strains of *Pseudomonas aeruginosa*. Another example is trehalolipids, generally produced by gram-positive bacteria such as *Mycobacterium*, *Corynebacterium*, and *Nocardia*. Apart from rhamnolipids and trehalolipids, examples of other glycolipid classes are sophorolipids, which are generally produced by *Candida* strains. The second group is lipopeptides, an example of which is surfactin, generally produced by *Bacillus* strains (Clements *et al.*, 2019).

Polymeric biosurfactants, fatty acids, phospholipids, and neutral lipids are some of the other types of biosurfactants. These include emulsions, grounds, liposans, and other polysaccharide protein complexes. In addition to being categorized according to their molecular structure, biosurfactants are further divided into two groups: low and high molecular weights. High molecular weight biosurfactants function as bioemulsifiers and can stick securely to a variety of surfaces. One thing that has a high molecular weight is an emulsion (emulsion stabilizer), which is made up of hydrophilic carbohydrates and hydrophobic fatty acids connected by O-esters or N-acyls. Meanwhile, low molecular weight consists of glycolipids and lipopeptides, which can reduce surface tension.



Biosurfactants have a more complicated structure than synthetic surfactants. This more complex structure makes biosurfactants the next generation of surfactants because they are more environmentally friendly and multifunctional. In contrast to biosurfactants, synthetic surfactants have neater hydrophilic and hydrophobic regions. The hydrophobic structure of chemical surfactants usually consists of linear alkyl groups because branched groups are more difficult to decompose, so they are rarely used. The hydrophilic structure forms the main basis for the classification of surfactants, namely anionic, cationic, non-ionic, and zwitterionic (Fenibo *et al.*, 2019).

The nature of biosurfactants, which are biodegradable and do not cause toxic effects, makes them a globally valuable product. One of them can be applied to the oil industry. The addition of biosurfactants to microbial enhanced oil recovery (MEOR) is able to recover trapped oil well. Biosurfactants can also be applied for the biodegradation of petroleum because of their amphiphilic nature, so that stable micelles are formed to increase the bioavailability of hydrocarbons (Johanes *et al.*, 2022).

When there are insufficient nutrients in the medium during the late exponential phase or the stationary phase, microorganisms synthesize biosurfactants both intracellularly and extracellularly. Bacteria can metabolize chemicals through partition, passive diffusion, assisted transport, or active transport when the compounds are aromatic or otherwise non-water soluble. But substances like naphthalene, anthracene, phenanthrene, polycyclic aromatic hydrocarbon (PAH), and other extremely hydrophobic substances are absorbed via diffusion, which is then followed by the synthesis of biosurfactants to enhance the hydrophobicity of the cell surface. Therefore, hydrocarbon-degrading bacteria have the potential to also produce biosurfactants (Kumar *et al.*, 2021).

To date, much research has been carried out on biosurfactant-producing bacteria. Biosurfactant-producing halotolerant and halophilic bacteria isolated from mangrove ecosystems have been reported to accelerate oil remediation in medium- to high-salinity environments. It has been reported that bacteria from the genera *Acinetobacter*, *Bacillus*, *Corynebacterium*, *Marinobacter*, *Pseudomonas*, *Rhodococcus*, and *Serratia* can make biosurfactants in oily land and sea environments. Although there has been a lot of research on biosurfactant-producing bacteria, the search for biosurfactant-producing bacteria in marine or saline environments is still ongoing. This is because the marine biosphere is so large that there are still many microorganisms that have not been explored. In addition, most of the production of broad-spectrum secondary metabolites is isolated from marine microorganisms (Pathania and Jana, 2020).

Biosurfactant production is influenced by various factors, including carbon source, pH, and salinity. Biosurfactant production in the growth of microorganisms is triggered when

there is a limitation of one or more components in the medium and the availability of hydrocarbons that do not mix with water in the growth medium. The carbon source that is widely used in biosurfactant production is olive oil because it has good emulsion stability in various bacterial strains. The greatest biosurfactant activity was demonstrated by *Paenibacillus* sp. 1C when grown in 2% (v/v) olive oil, resulting in a surface tension of 33 mN/m. When grown in 1% (v/v) olive oil, *Pseudomonas* sp. W10 can also create ramnolipids, which lower surface tension by 32 mN/m (Sanches *et al.*, 2021).

In addition to the carbon source, the growing medium's pH affects the synthesis and function of biosurfactants. A healthy pH is necessary for the development of microorganisms' cells and the synthesis of secondary metabolites like biosurfactants. Optimal biosurfactant production for each strain of microorganism is different; generally, it is optimal at a pH close to 7, but some reports show a slight increase in pH up to 8.5 (Andry *et al.*, 2023). Biosurfactant production from *Paenibacillus* sp. D9 is optimal at pH 7. The emulsification activity of *Rhodococcus erythropolis* AQ5-07 was reported to be seen in the pH range of 7–8, but the highest activity was obtained at pH 7.5. The effect of media pH on the emulsification index (IE24) also depends on the carbon source used. Biosurfactant from *Pseudomonas aeruginosa* ATCC 15442 is optimal at pH 7, with the highest IE24 of 27% when cultured with diesel fuel as the carbon source, whereas when using crude oil, it is optimal at pH 6, with an emulsification index of 50% (Buonocore *et al.*, 2020).

Another factor that influences biosurfactant production activity is salinity. Salinity is related to the characteristics of microorganisms in the environment, such as microorganisms from the marine environment that have halophilic properties (Heryadi *et al.*, 2023). *Bacillus stratosphericus* FLU5 isolated from seawater in the Port of Sfax, Tunisia, was stable when the NaCl concentration was increased by 0–120 g/L and obtained a surface tension of around 33–34 mN/m, but was disturbed when the concentration was increased by 175–200 g/L NaCl. *Streptomyces* sp. B3 isolated from West Coast sediments in India produced the highest activity at 4.2% (w/v) NaCl with an emulsification index of 75%. Because biosurfactants don't break down when there is a lot of salt in the water, they can also be used to clean up hydrocarbon-contaminated seas (Chebbi *et al.*, 2017).

Mangrove ecosystems are unique and vital coastal environments, found between freshwater and marine areas, that are ecologically rich in biodiversity. The specialty of this ecosystem lies not only in its visual beauty but also in its important role in maintaining the balance of the coastal environment (Humaida *et al.*, 2018). The high productivity of mangroves indicates their important role in providing habitat for various species, from fish to migratory birds. Mangroves act as a natural filter to control runoff entering the sea. Due to the mangrove roots' natural filtering process, the complex root



system of mangrove trees serves as a retainer for mud and sediment, making the water flowing into the sea clearer. Apart from that, mangroves are also known as significant carbon stocks. Mangrove plants are able to absorb carbon dioxide (CO₂) from the atmosphere and store it in their own tissues, helping to reduce the impact of greenhouse gases that trigger climate change (Hamzah and Harijati, 2023).

The role of mangroves as sediment stabilizers is very important in maintaining coastal stability. The strong roots of mangroves help reduce the rate of coastal erosion due to sea waves and wind. In doing so, they provide protection for coastal areas from potentially damaging erosion. Mangrove ecosystems also play an important role in nutrient cycling (Chen *et al.*, 2017). Fallen leaves and organic material from mangroves make a major contribution to providing a source of nutrition for marine organisms and creating a fertile environment for various forms of marine life. Finally, the role of mangroves as coastal protectors cannot be ignored. They act as natural fortifications, reducing the impact of waves and storms that can damage coastal areas and surrounding infrastructure (Clements *et al.*, 2019). With the many roles played by the mangrove ecosystem, it is important for us to maintain and protect its existence. The sustainability of mangroves is not only for their own natural life but also affects the balance of the global environment as a whole.

MATERIALS AND METHODS

This research is descriptive research for screening and characterization of biosurfactants, while experimental research is for optimizing biosurfactant production. There were two stages of screening: the first stage used the drop collapse method (DCM), and the second stage measured the emulsification index for 24 hours (IE24) with 10% SDS as a positive control. The method used in biosurfactant optimization is self-directing optimization, namely testing the optimization factors one by one and discarding the factor that gives the least satisfactory results. Optimization in this research used a completely randomized design (CRD). The parameter used in biosurfactant optimization is IE24.

There are three test factors for optimization, with each factor consisting of five levels. Factor I is the concentration of the carbon source, which consists of 4–6% (v/v) with an interval of 1%. Factor II is pH, which consists of 6–8 with an interval of one. Factor III is salinity, which consists of 2–4% (w/v) with an interval of 1%. The level of factor I with the highest IE24 will be used for factor II, and the best level of factor II will be used for factor III. Each optimization experiment was carried out in triplicate, and the average value was calculated. Biosurfactant characterization uses two methods, namely fourier transform infrared (FTIR) to see the functional groups formed and liquid chromatography mass spectrometry (LCMS) to predict the molecular structure of the biosurfactant produced.

This research consists of several stages, starting with preparing materials and tools, making media, creating a working culture, and starting. The next stage is primary and secondary screening, the best results of which will be selected for the optimization stage. Optimization was carried out based on carbon source concentration, pH, and salinity. Biosurfactants with the best activity are extracted for use in the characterization stage using FTIR and LCMS. The final stage is the analysis of the data obtained from the results of screening, optimization, and characterization.

The materials used in this research were Nutrient Broth (NB), Bacto Agar, yeast extract, NaCl, KH₂PO₄, K₂HPO₄, (NH₄)₂SO₄, FeSO₄·7H₂O, MgSO₄·7H₂O, CaCl₂·2H₂O, MnSO₄·H₂O, SDS (Sodium Dodecyl Sulfate), distilled water, NaOH, HCl, olive oil, n-hexadecane, methanol, chloroform, and ammonium acetate.

The equipment used in this research was Laminar Air Flow (LAF), refrigerator, autoclave, microwave, pH meter, UV-Vis spectrophotometer, stereo microscope, centrifuge, incubator, shaking incubator, vortex, analytical and precision balance, erlenmeyer, test tube, stopper, 96-well microplate, separating funnel, ose, spirit burner, lighter, gloves, tissue, aluminum foil, cling wrap, micropipette 1000 µL and 1–10 µL, microtip, sterile syringe filter 0.2 µm, 20 ml syringe, cryovial, 15 ml and 50 ml centrifuge tubes, 2 ml microtubes, rotary evaporator, Fourier Transform Infrared spectroscopy (FTIR) and Liquid Chromatography Mass Spectrometry (LCMS).

RESULTS AND DISCUSSION

The results of primary screening using the drop collapse method showed that there were 25 isolates that gave positive results as biosurfactant producers. A total of 20 isolates produced slightly flat droplets, while the other 3 isolates produced quite flat droplets. Isolate *Prescottella equi* NNA 0-4, *Thioclava* sp. NNA 0-6, and *Pseudomonas aestusnigri* NNA 0-8 had flatter droplet shapes compared to the other isolates but not flatter than the positive control (SDS 10%). This indicates that the three isolates have the ability to reduce interfacial tension. The flatter the droplet shape, the better the interfacial tension.

When the emulsification index (IE24) was used for secondary screening, it showed that up to 10 isolates could mix with n-hexadecane to make an emulsion. *Pseudomonas aestusnigri* NNA 0-8 was the best isolate for emulsifying n-hexadecane, with an IE24 value of $40 \pm 0.92\%$, but not better than the positive control (SDS 10%), which gave an IE24 value of $65 \pm 2.2\%$. The following isolates showed positive results in the first screening: *Exiguobacterium profundum* TWR 0-1.1, *Marinobacter* sp. TWR 100-3, *Rhodococcus ruber* TWR 100-4; *Thioclava nitratireducens* NNA 0-2; *Rhodococcus ruber* NNA 0-4; *Thioclava* sp. NNA 0-6; *Rhodococcus ruber* NNA 0-9; *Thioclava nitratireducens* NNA 50-4; *Thioclava* sp. NNA 50-5; *Rhodococcus ruber* NNA 50-8; and *Rhodococcus* sp.



NNA 100-3; *Thioclava* sp. NNA 100-4; and *Rhodococcus gannanensis* NNA 100-5. This is because the biosurfactant isolates are not effective in stabilizing the emulsion with n-hexadecane. Biosurfactants from *Ochrobactrum anthropi* HM-1 and *Citrobacter freundii* HM-2 are effective in emulsifying diesel oil, crude oil, and used cooking oil, but are not effective in emulsifying hexadecane and kerosene.

The results of primary and secondary screening showed that *P. aestusnigri* NNA 0-8 had the best ability to produce biosurfactants among all isolates. However, these results do not support the potential of the strain as a prospective biosurfactant-producing isolate. One of the criteria for selecting biosurfactant-producing isolates is those that can produce IE24 above 52%. Therefore, the culture conditions of *P. aestusnigri* NNA 0-8 need to be optimized to increase biosurfactant productivity.

Pseudomonas aestusnigri NNA 0-8 can utilize olive oil with a concentration of 4-7% (v/v) as a carbon source to produce biosurfactants. Biosurfactant activity began to appear on the 3rd day at olive oil concentrations of 5 and 6%, while activity at concentrations of 3 and 7% only began to appear on the 4th day. The five olive oil concentrations tested had varying IE24 values. The results of the ANOVA test showed that the olive oil concentration of 3-7% on days 5-10 had a significant effect on the IE24 value. The highest IE24 value was found at the 5% concentration on day 6, amounting to $55 \pm 2.2\%$. The IE24 value is significantly different from concentrations of 4, 6, and 7% in the Duncan test results.

An increase in biosurfactant activity occurred as the olive oil concentration increased from 3-5%, then decreased at higher concentrations. Increasing the concentration of olive oil as a carbon source from 0.5% to 4% has also been reported to increase the IE24 biosurfactant of *Haloarcula* sp. IRU1 from 23% to 35%. The concentration of carbon sources is related to the C/N ratio required in the biosurfactant production medium. A high carbon-to-nitrogen ratio will limit bacterial growth and thus support biosurfactant production. The nitrogen concentration in this study was not changed (3 g/L (NH₄)₂SO₄ and 0.3 g/L yeast extract), so only the effect of the carbon source concentration was considered. When the nitrogen has been used up, biosurfactants will be produced to increase the solubility of the oil. When the oil is in the dissolved phase, the lipid components of the oil will induce the hydrophobic part of the biosurfactant during the carbon metabolism process.

Although increasing the concentration of olive oil can increase biosurfactant activity, olive oil concentrations of more than 5% actually decrease biosurfactant activity. This is because high amounts of oil have strong hydrophobic interactions, making it difficult for surfactant molecules to form micelles. As a result, the oil is still large in size, so the solubility of the oil is reduced and surfactant biosynthesis is hampered. The biosurfactant activity of *P. aestusnigri* NNA 0-9 can be seen on day 3 at pH 8 and 9. ANOVA test results

show that pH 8 and 9 from day 3, 11, 12, 13, and 14 have a significant effect on the IE24 value. The highest IE24 value was obtained at pH 7 on day 6, namely $58 \pm 1.6\%$. pH 8 also had the highest IE24 value on day 6 of $56 \pm 2.5\%$. The two IE24 values (pH 7 and 8 on day 6) were not significantly different based on the Duncan test results.

Other results were shown at pH 5 and 6, where no biosurfactant activity was found for 14 days. The absence of biosurfactant activity at pH 5 and 6 indicates that *P. aestusnigri* NNA 0-8 is unable to produce biosurfactant at an acidic pH when cultured with MSM and olive oil as a carbon source. Using a pH that tends to be more alkaline can produce good biosurfactant activity in *P. aestusnigri* NNA 0-8. This is related to the use of olive oil with a high concentration, namely 5%, so the fatty acid content is high. *Pseudomonas aeruginosa* M4 biosurfactant is optimum at pH 8 when cultured with MSM sourced from soybean oil carbon, which is high in fatty acids. The use of a higher initial pH allows the fermentation system to tolerate high fatty acid concentrations. The results of the ANOVA test showed that the NaCl concentration of 2-5% on days 2 to 4 had a significant effect on the IE24 value. However, NaCl concentrations of 2% on day 3 had IE24 values that were not significantly different from each other. The highest IE24 value was found at 1% salinity on day 3, amounting to $47.5 \pm 4.4\%$. Biosurfactant activity can be seen on day 2 for NaCl concentrations of 2-4%. Meanwhile, at 5% salinity, it can only be seen on the 4th day, but after the 4th day, there is no activity. The salinity graph of 1-4% NaCl increases until the 3rd day and then decreases after that.

Pseudomonas aestusnigri NNA 0-9 was still able to maintain biosurfactant activity at 2% NaCl, but decreased significantly when the NaCl concentration was above 3%. This indicates that *P. aestusnigri* NNA 0-9 has a low level of tolerance to salinity above 3% to produce biosurfactant. This condition is related to *P. aestusnigri* NNA 0-9, which was isolated from a location with a salinity of 25.2 ppt, so it cannot tolerate high salinity. *Pseudomonas aeruginosa* NAPH6 isolated from seawater in the Port of Sfax, Tunisia, with a salinity of 34.2 g/l also had constant biosurfactant activity at 0-4% NaCl and decreased further when the NaCl concentration was increased.

Pseudomonas aestusnigri NNA 0-9 in this study had a low level of salinity tolerance, characterized by a decrease in biosurfactant activity with the addition of 1-4% NaCl. These results are different from the original habitat of *Pseudomonas aestusnigri*, which has the original name *Halopseudomonas aestusnigri*, because it can grow in 2-8% NaCl. Therefore, it is possible that the *P. aestusnigri* NNA 0-9 isolate is a strain adapted to mangrove habitats with low salinity. Increasing the NaCl concentration can also cause a decrease in the critical micelle concentration of some surfactants, thereby inhibiting their distribution in solution. This is known as the salting-out effect, where the hydrophobic part of the biosurfactant will



settle out. As a result, biosurfactant activity decreases at high salinity levels. The salinity treatment in this study had a lower IE24 value than the treatment without salinity. Therefore, salinity treatment was not continued at the optimization stage. Incubation time can range from several hours to several days to obtain maximum productivity. Based on the ANOVA test, the difference in incubation time for 12 days had a significant effect on the IE24 value. The best incubation time (6th day) for factor I (5% olive oil concentration as a carbon source) was significantly different from other incubation days. Meanwhile, the best incubation time (6th day) for factor II (pH 7) was not significantly different from the 7th and 8th days. In this study, *P. aestusnigri* NNA 0–9 had the best time period on day 6 in producing biosurfactant both in factor I (5% olive oil as a carbon source) and factor II (pH 7), with the respective emulsification indexes of $55 \pm 1.5\%$ and $58.2 \pm 1.1\%$. The same results were obtained from previous research: the production of *Pseudomonas aeruginosa* A2 biosurfactant could reach its highest level after 6 days of incubation. Biosurfactant production tends to take a long time because it is a secondary metabolite that is generally produced during the stationary phase when nutrients are limited. In addition, the biosurfactant regulatory mechanism is controlled by quorum sensing, so it takes time to reach a certain cell density in minimal media.

The biosurfactant extract of *P. aestusnigri* NNA 0–9 was examined for its character using FTIR to see the functional groups of the biosurfactant compound produced. Based on a comparison of the FTIR spectrum in this study with the reference, the biosurfactant *P. aestusnigri* NNA 0–9 refers to its rhamnolipid character. Most of the *Pseudomonas* genus is known to produce glycolipid biosurfactants, namely rhamnolipids. Rhamnolipids consist of a hydrophobic tail containing one or two fatty acids linked by a carboxyl group to one or two rhamnose molecules.

The composition of rhamnolipid functional groups was identified from the peaks that appeared in the FTIR spectrum. Absorption at peak 3312.15 cm⁻¹ shows the stretching vibration of the hydroxyl group O-H, indicating the presence of polysaccharides. The peak between 3000 and 2700 cm⁻¹ (C-H group), which is generally found in rhamnolipids, was not detected. This peak was not detected, presumably because the extraction method was different. The difference in solvents used in rhamnolipid extraction resulted in several functional groups not being detected. Peak 2130.5 cm⁻¹ indicates the presence of CH₃, which is bound to the rhamnose and lipophilic ring in the glycopeptide. Peak 1640.9 cm⁻¹ indicates the stretching vibration of the C=O carbonyl group of the lipid chain structure. Peaks 1087.6 cm⁻¹ and 1020.6 cm⁻¹ indicate C-O-C bonds, confirming the existence of bonds between carbon atoms and hydroxyl groups in the rhamnose structure. Peaks in the fingerprint region below 1200 cm⁻¹ represent various unallocated C-H, C-O, and CH₃ bonds.

LCMS analysis of the biosurfactant *P. aestusnigri* NNA 0–9 was carried out in positive ion mode for identification of its structural constituents. Peaks detected in the LCMS data were compared with previous literature. There were three rhamnolipid peaks detected in the biosurfactant extract of *P. aestusnigri* NNA 0–9, namely m/z 505, 607, and 640. Peak m/z 604 indicated the presence of a mono-rhamnolipid structure with a homologue [Rha-C11-C16+H]⁺, [Rha-C12-C15+H]⁺, and [Rha-C13-C14+H]⁺ and m/z 639 with homologs [Rha-C14-C16:2-H]⁺, [Rha-C15-C15:2-H]⁺, [Rha-C14:2-C16-H]⁺, and [Rha-C15:2-C15-H]⁺. Di-rhamnolipid was detected at m/z 507 with the homologue [Rha-Rha-C12:1+H]⁺.

The dominant rhamnolipid representative at m/z 604, namely [Rha-C11-C16+H]⁺, was formed by adjusting m/z 340, which is the most dominant lipid chain. Meanwhile, the molecular structure of homologues at other m/z values could not be established because other lipid chain peaks were not detected. Based on relative intensity, the dominant biosurfactant produced by *P. aestusnigri* NNA 0–9 is mono-rhamnolipid with homologs Rha-C11-C16, Rha-C12-C15, and Rha-C13-C14, characterized by an intensity of 100%. The di-rhamnolipid biosurfactant that the *Pseudomonas* genus typically produces is dominant. However, mono-rhamnolipids have also been found to be the dominant component in several *Pseudomonas* genera. For example, it was found in *Pseudomonas aeruginosa* strain ATCC 9027 with a mono-rhamnolipid to di-rhamnolipid ratio of 24:1. *Pseudomonas gessardii* also dominantly produces 16 mono-rhamnolipids.

The various compositions of rhamnolipids in both the rhamnose and lipid groups influence their properties and applications. Mono-rhamnolipids are reported to be more effective in reducing surface tension and have a higher IE24. The antibacterial activity of mono-rhamnolipid is also better than that of di-rhamnolipid. In addition, mono-rhamnolipid was more effective in dissolving PAH than di-rhamnolipid. This is because mono-rhamnolipids only consist of one rhamnose group, so their lipophilic properties are better. The presence of better lipophilic properties causes longer release of PAHs with mono-rhamnolipids, so the level of biodegradation of di-rhamnolipids is better compared to mono-rhamnolipids. Besides that, both are equally effective in MEOR applications. The presence of a mixture of mono- and di-rhamnolipids in the *P. aestusnigri* NNA 0–9 culture makes this isolate prospective for use in various applications, such as bioremediation, MEOR, pharmaceuticals, and various other industries.

Conclusion: Research focused on biosurfactant production by the bacteria *P. aestusnigri* NNA 0–9, originating from the mangrove ecosystem, has revealed optimal conditions for producing this compound. Through a series of experiments, it was found that the best conditions for making biosurfactant were 5% olive oil as a carbon source, a solution pH of 7 without any extra NaCl, and an incubation period of 6 days.



The molecular structure analysis method using the FTIR technique confirmed that the resulting compound was rhamnolipid. Further results from this analysis show structural variations in the form of mono-rhamnolipids, such as Rha-C11-C16, Rha-C12-C15, Rha-C13-C14, Rha-C14-C16:2, Rha-C15-C15:2, Rha-C14:2-C16, and Rha-C15:2-C15. In addition, using the LCMS technique, di-rhamnolipid with the structure Rha-Rha-C12:1 was found. The conclusions of this study provide a detailed description of the optimal parameters required for biosurfactant production while also providing identification of the molecular structure produced by the bacterium *P. aeruginosa* NNA 0-9 from mangrove. These findings provide a strong foundation for further development regarding the use of biosurfactants in industrial and environmental applications. In the optimization stage, statistical approach methods such as Response Surface Methodology (RSM) need to be used to obtain an optimization model. The dependent variable in optimizing biosurfactant production can also be added to the values of surface tension, interfacial tension, surface contact angle, and critical micelle concentration (CMC).

Authors' contributions: All of authors are participate in experiment, writing and drafting of manuscript similarly.

Funding: None.

Ethical statement: this experiment is not include any handling for animal and plant. Whereas condition of experiment was completely environment-friendly.

Availability of data and material: Raw data can be available when journal requests from corresponding author.

Consent to participate: None

Consent for publication: authors have not any conflict of interest.

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